PROTEINS IN MEMBRANE MIMETIC SYSTEMS

Insertion of Myelin Basic Protein into Microemulsion Droplets

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ABSTRACT The insertion of myelin basic protein into microemulsion droplets of sodium bis (2-ethylhexyl) sulfosuccinate (AOT) has been studied by quasi-elastic light scattering. Measurements were made at both low and high molar ratios of water to surfactant, as a function of protein occupancy. The hydrodynamic radii of filled and empty droplets were experimentally evaluated. These were compared to values calculated using a water shell model of protein encapsulation, and excellent agreement was obtained. At low molar ratio of water to surfactant (w_0 = 5.6), the hydrodynamic radius of filled droplets is significantly larger than the radius of empty ones. Under these conditions, about three empty (water-filled) droplets are required to build up a droplet of sufficient size to accommodate a single protein molecule. At maximum solubilization, which occurs at w_0 = 5.6, a small fraction of droplets are found containing protein aggregates. In contrast, results at high values of w_0 (22.4) reveal radii for empty and occupied droplets of comparable dimension, and the absence of aggregates. The results are discussed in terms of the model and the mechanism of interaction of this protein with the aqueous interfaces provided by these membrane-mimetic systems.

INTRODUCTION

Aggregates of surfactant in organic solvents assemble into microemulsion droplets in the presence of water (1). The unusual physical properties of the entrapped water, markedly different from bulk water but similar in several respects to the behavior of biological interfacial water, have drawn the attention of many investigators in the last few years (2). Moreover, the ability of reverse microemulsions to solubilize peptides (3), enzymes (4), nucleic acids (5), or integral membrane proteins (6) is now well documented, as are the resulting structural and catalytic properties (7).

To account for the solubilization of hydrosoluble proteins, Bonner et al. (8) have proposed a model in which the protein encapsulated in the aqueous core of droplet is separated from the hydrocarbon solvent by a monolayer of surfactant molecules and a shell of water. The water-shell model has received satisfactory experimental support with respect to several proteins at different molar ratios of water to surfactant w_0.

Myelin proteins from the central nervous system (CNS) display distinctive properties in microemulsions made of sodium *bis* (2-ethylhexyl) sulfosuccinate (AOT), isooctane, and water (6). One of these is myelin basic protein (MBP), a 169-residue water-soluble protein, whose sequence has been determined by Eylar (9), and which constitutes 30% of the total protein of the multilamellar myelin sheath. The protein is located between the cytoplasmic apposition of surfaces of the oligodendrial membrane and has been extensively studied (10). In situ, MBP is localized in the aqueous interstitial space sandwiched between two lipid bilayers. If one considers only those lipid layers adjacent to the aqueous space, this is equivalent to its location in an inverted bilayer environment with the polar heads of the lipids in contact with the interfacial water and the hydrophobic tails inside the hydrocarbon bilayers. Therefore, with respect to this protein, the aqueous spaces of myelin bear a resemblance to its environment in microemulsion droplets dispersed in hydrocarbon solvents.

In this paper, we report the study of the insertion of MBP into microemulsion droplets starting at low water-to-surfactant molar ratio. Using quasi-elastic light scattering, we show that under such conditions at maximum solubilization the hydrodynamic radius (R_f) of protein-containing droplets is significantly larger than the radius R_e of water-filled droplets. We have also determined the number of water-filled micelles necessary to build up a droplet of a prerequisite size to accommodate a single protein molecule (M_r = 18,500). The resulting redistribution of surfactant and water molecules probably originates from a competition between surfactant and myelin protein for interfacial water layers.

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MATERIAL AND METHODS

Materials

Sodium bis (2-ethylhexyl) sulfosuccinate (AOT), a generous gift from the Cyanamid Co. (France), was purified according to Wong et al. (11) and carefully dried in vacuo. The purity of each batch was checked according to the procedures recommended by Luisi et al. (12). Isooctane (Uvasol grade) was purchased from Merck (Darmstadt, Federal Republic of Germany).

MBP was extracted and purified from bovine white matter, using the method of Deibler et al. (13), and lyophilized.

Preparation of Microemulsions

The samples were prepared by addition of measured volumes of isooctane to dry, preweighed amounts of AOT. The desired volumes of double distilled water (vol/vol) were added using Hamilton syringes and the samples shaken until optically clear. Dried, preweighed quantities of MBP were added and left for 2 h at room temperature. Dissolution was achieved by gentle shaking followed, when necessary, by 2 min of sonication in a Branson sonicator (Branson, Geneva, Switzerland) sonicator. A final centrifugation (model GR2000S; Jouan, Paris, France) at 5,000 rpm removed the undissolved protein.

Two series of microemulsions were prepared. The AOT concentration was 50 mM, the water to surfactant molar ratio was $w_0 = 5.6$ for the first series, and $w_0 = 22.4$ for the second. The samples were analyzed and studied at constant temperature $t = 20 \pm 0.01$°C in a thermostated bath (Haake, Karlsruhe, Federal Republic of Germany).

In the rest of the paper f is defined as the ratio of the number of protein molecules to the number of droplets initially present in the system.

Absorption Measurements

The absorption spectra were measured on a Cary model 118 spectrophotometer using a molar extinction coefficient of 1.07×10^4 for MBP in water or in reverse micelles, at 278 nm.

Light Scattering Measurements

Photon-beating spectroscopy (14) has been used to measure the hydrodynamic radius of microemulsion droplets. The light source is an Argon ion laser (model 165; Spectra Physics, Mountain View, CA). A typical value of the intensity impinging on the sample was 0.2 W at $\lambda = 514.5$ nm.

The self-correlation function of the scattered light has been recorded over a range of angles from 30° to 150° using an EMI 9558QB photomultiplier followed by a counting device. The Malvern 4 bits × 4 bits digital correlator was interfaced to a desk computer (Hewlett-Packard 9835; Hewlett-Packard Co., Palo Alto, CA).

Due to the low scattering power of some samples, occasional dust particles could seriously disturb the results. This problem has been solved by introducing a check procedure. At the end of each run (10^6 sweeps), the baseline is calculated and compared with the value measured by using delay channels. If some discrepancy occurs ($>5 \times 10^{-3}$ of the signal amplitude), the data are rejected. If not, they are stored in the computer. At least 100 runs per experiment are collected. A nonlinear program using Marquardt procedure (15) is used to fit the experimental curve to analytical formulae, as discussed later.

MODEL FOR PROTEIN SOLUBILIZATION AND ITS CONSEQUENCES FOR LIGHT SCATTERING EXPERIMENTS

The results will be presented and discussed in terms of a model of Bonner et al. (8). The main features of this model are summarized below.

The initial microemulsion is a dispersion of spherical droplets ("empty" droplets). The radius R_w of the water core depends on the water to surfactant molar ratio, w_0, and increases with it. The hydrodynamical radius R_e is $R_e = R_w + l$, where l is the length of the AOT molecule ($l = \sim 12$ Å [16]). (The hydrodynamic radius, R_e, is actually measured by light-scattering experiments. R_e includes the water core as well as the tails of the surfactants.) The protein volume is V_p. For convenience, a radius R_p ($R_p = (3V_p/4\pi)^{1/3}$) is introduced, without making any assumption about the actual protein shape. Each protein molecule is supposed to be enclosed in the water core of a large droplet ("filled" droplet) built up with one protein molecule and n empty droplets. The n value is fixed by the following constraints: (a) the area per surfactant polar head is constant (17); (b) the w_0 ratio in the droplet is the same as in the initial microemulsion (8).

Let R_i be the radius of the core of the filled droplets. The constraints are expressed by

$$R_i = (R_p^3 + nR_w^3)^{1/3}, \tag{1a}$$

which is the volume conservation of the microemulsion droplet inner core

$$R_i = n^{1/2} R_w, \tag{1b}$$

which is the surface conservation of the microemulsion droplet. The hydrodynamical radius of the full droplets is $R_f = R_i + l$. Note that the size of the remaining empty droplets stays unchanged as a consequence of the second constraint.

Discussion of the Model Validity

The model assumes that there are enough empty droplets to accommodate all the proteins present, the filled droplets being made of n empty droplets. This means that the average number f of protein molecules per microemulsion droplet has to be smaller than $1/n$, which appears as a threshold value for f. For $f > 1/n$, it is no longer possible to insert each one protein molecule per one droplet: droplets containing two proteins or more will be formed and protein aggregation will take place (a distinction is made between droplets containing one or two protein molecules and aggregates containing greater numbers of them).

Predicted Correlation Functions

Without protein present, the microemulsion is a dispersion of spherical droplets. For a monodispersed system, the correlation function decay is monoexponential, with a time constant $\tau = 1/2Dq^2$, where D is the diffusion coefficient of droplets and q the scattering wave vector is expressed by

$$q = \frac{4\pi n_0}{\lambda} \sin \frac{\theta}{2},$$

where θ is the scattering angle, n_0 the sample refractive index, and λ the wavelength of light in vacuo ($\lambda = 514.5$ nm). For dilute systems, the hydrodynamic radius R_e of droplets can be deduced from the Stokes-Einstein formula $R_e = kT/6\pi\eta D$, where k is the Boltzmann constant, T the absolute temperature, and η the isooctane viscosity. For moderately polydispersed systems, the correlation function is expected to deviate from the exponential shape according to

$$g_2(t) = \{Ae^{-t/\tau + p/2(t/\tau)^2}\}^2 \tag{2}$$

The polydispersity index p is related to the width of the size distribution function (18). As previously, τ scales as q^{-2} and a mean hydrodynamical radius R can be obtained. At low protein concentration ($f < 1/n$), empty droplets are expected to coexist with filled ones.

In the following section, the radii R_e (empty droplets) and R_f (filled droplets) will be found to be comparable. A two exponential fit is then not convenient. A one exponential fit with some degree of polydispersity will be better. Therefore, we expect a good agreement with Eq. 2.

Note that the polydispersity index accounts for the presence of two different radii, R_e and R_f, and for some size distribution around these values, due to the intrinsic polydispersity of the initial microemulsion. The

hydrodynamical radius, R, is an average of the radii R_e and R_f, weighed by the relative amount of empty and filled droplets and their scattering power and can be calculated as a function of f knowing R_e, R_f, and n (see Appendix). At higher protein concentration ($f \geq 1/n$), associations will occur, i.e., droplets containing two or three protein molecules and, very rapidly, larger aggregates. These are expected to be rather polydisperse. A slow, nonexponential decay is expected to occur. Since it is not meaningful to fit the correlation function with two exponentials both including polydispersity, the polydispersity of aggregates only will be taken into account.

Thus a good model for the correlation function will be

$$g_2(t) = \{A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2 + p/2(t/\tau_2)^2}\}^2 \quad (3)$$

with $\tau_2 \gg \tau_1$, both τ_1 and τ_2 scale with q^{-2}. From these values, the hydrodynamical radii, R_1 (average value for droplets) and R_2 (aggregates), are calculated.

RESULTS

Microemulsions without Proteins

The correlation function is fairly well fitted to a single exponential. As expected, the fit is improved and the systematic deviations suppressed by introducing a moderate polydispersity. The hydrodynamical radii measured at w_0 values of 5.6 and 22.4 are 29 and 50 Å ± 1 Å, respectively, with a polydispersity index p of 0.15 and 0.20. These radii are in good agreement with the measurements by Zulauf and Eicke (16). Theoretical estimations of the polydispersity due to thermal fluctuations in AOT microemulsions have been given by S. Safran (personal communication), in satisfactory agreement again with the measured index.

Protein Containing Microemulsions at $w_0 = 5.6$

Two different situations have been observed, as expected from the model. Figs. 1 and 2 present the light-scattering data and the fits of the correlation function. (*a*) For $f <$ 0.20, the correlation function fits well to Eq. 2 (see Fig. 1), from which R is deduced. p increases from 0.15 to a maximum value of 0.20 at $f = 0.09$. (*b*) for $f \geq 0.25$, a second much slower time decay becomes apparent (see Fig. 2). A good fit of the correlation function to the equation (3) is obtained, which allows calculation of the radii R_1 and R_2,

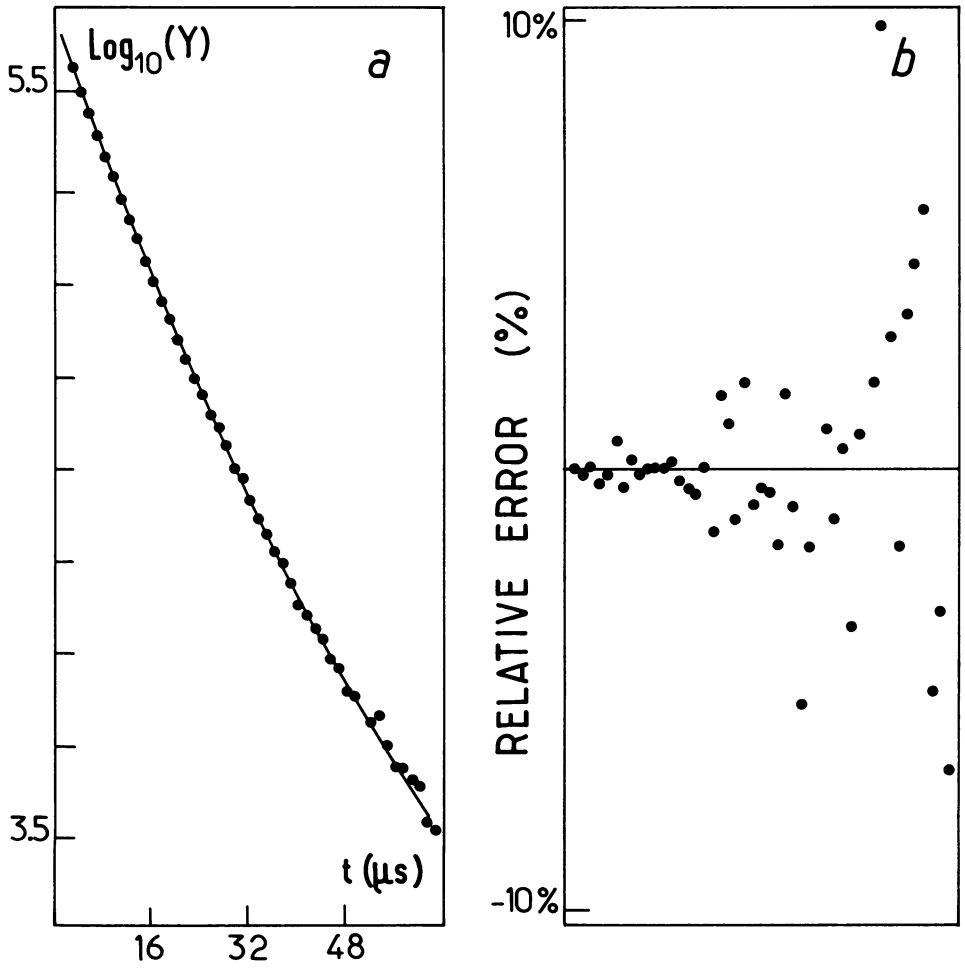

FIGURE 1 (*a*) Semi-logarithmic plot of the intensity autocorrelation function Y v. time for $w_0 = 5.6$ and $f = 0.05$. ●, experimental. ___, theoretical points. (Y in arbitrary units.) (*b*) Relative errors, $(Y_{exp} - Y_{th})/Y_{th}$, vs. time for the same system.

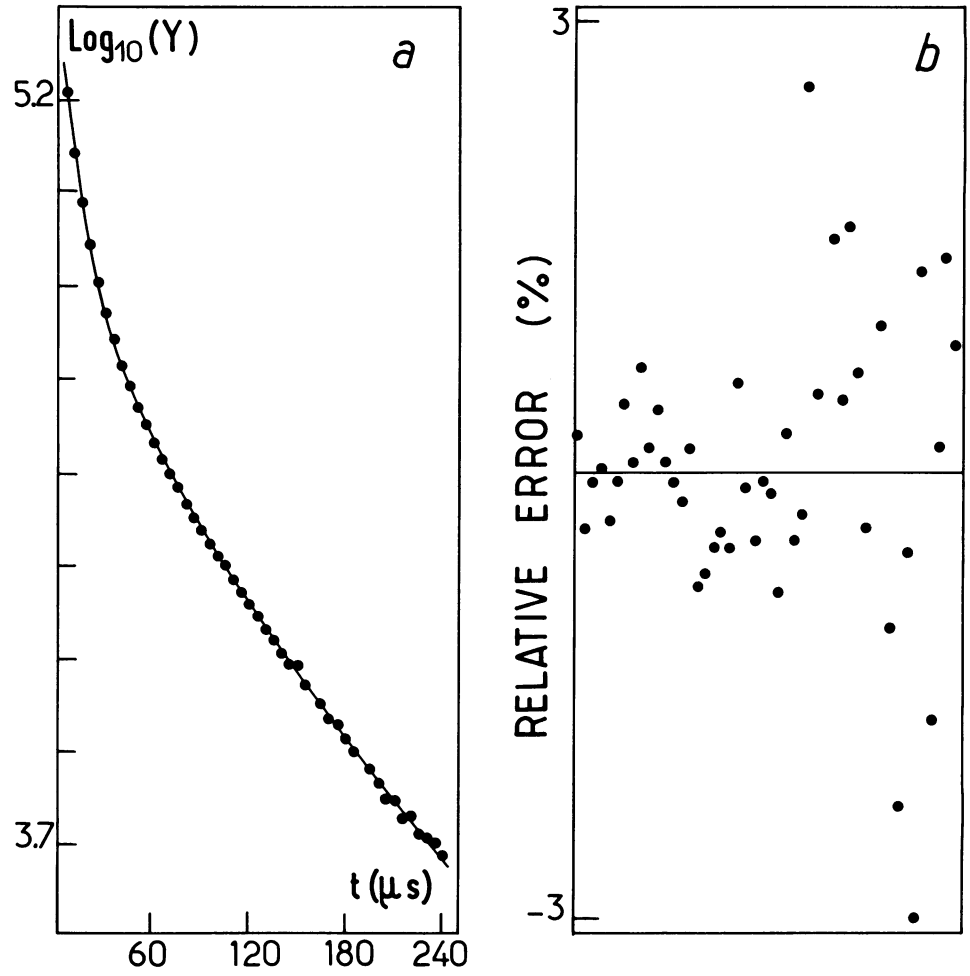

FIGURE 2 (*a*) Same as Fig. 1 *a*, but for $f = 0.34$ (*b*) Same as Fig. 1 *b*, but for $f = 0.34$.

for droplets and aggregates, respectively. Attempt to fit the correlation function to simpler formulae failed in this case, leading to significantly poorer results with systematic deviations.

In Fig. 3, the hydrodynamic radii R (from Eq. 2, for $f \leq 0.20$) and R_1 (from Eq. 3, for $f \geq 0.25$) are plotted vs. f. The occurrence of a plateau in the R_1 values suggests that the hydrodynamic radius R_f is close to 43 Å. Using the value of $R_e = 29$ Å (from which the radius of the water core is calculated: $R_w = 17$ Å) and the value of the radius of the filled droplet $R_f = 43$ Å (inner radius $R_i = 31$ Å), one can compute the values of n and R_p using Eq. 1 a and b. These values are 3.3 for n (average number of empty micelles necessary to build up a filled one) and 24 Å for the protein radius R_p.

No quantitative analysis of the size of aggregate can be made at present. In an attempt to reverse the aggregates, we have diluted microemulsions at high protein concentration, yet the aggregates remained present at the low protein concentration thus obtained. From the irreversible character of the aggregation process, it may be expected that only the order of magnitude of R_2 ($R_2 \sim 600$ Å) is significant. It has to be pointed out that the concentration of aggregates, obtained from the relative weight of the two times of the correlation function, was always found very low, in the range of 10^{-4}–10^{-6} times the concentration of filled droplets.

The mean radius, R, of droplets can also be calculated vs. f using the formulae given in the Appendix. It agrees quite satisfactorily with experimental points (see Fig. 3). The limit value of f (equal to $1/n = 0.3$) compares very well to experimental results. Indeed, some aggregates are formed even for $f \approx 0.25$, but the irreversibility of aggregation process causes the limiting value of f to be only approximative. The high value of R_1 at $f = 1$ suggests that droplets, including several protein molecules, actually exist. (We may note that a droplet with two protein molecules would have a hydrodynamic radius ~50 Å, provided they exist in a compact association. But at this point, we have no facts to support this assumption.) The results at $w_0 = 5.6$ are thus in good agreement with the model. Moreover, the calculated protein radius R_p is 24 Å, which agrees with the minimum value reported by Martenson in gel filtration experiments (19).

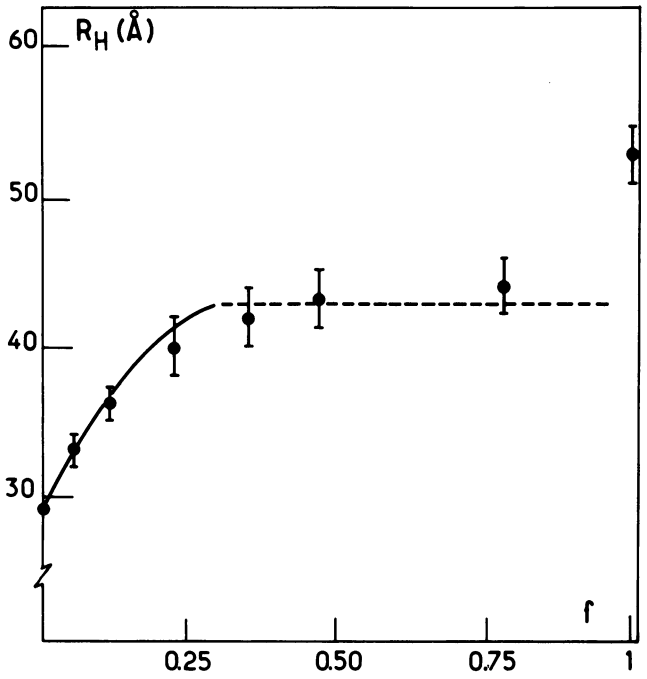

FIGURE 3 Hydrodynamic radius of droplets vs. protein occupancy factor f, for $w_0 = 5.6$; ● are the experimental values. The continuous curve is deduced from the model. The dotted line, R_H value for droplet with one protein ($R_H = 43$ Å).

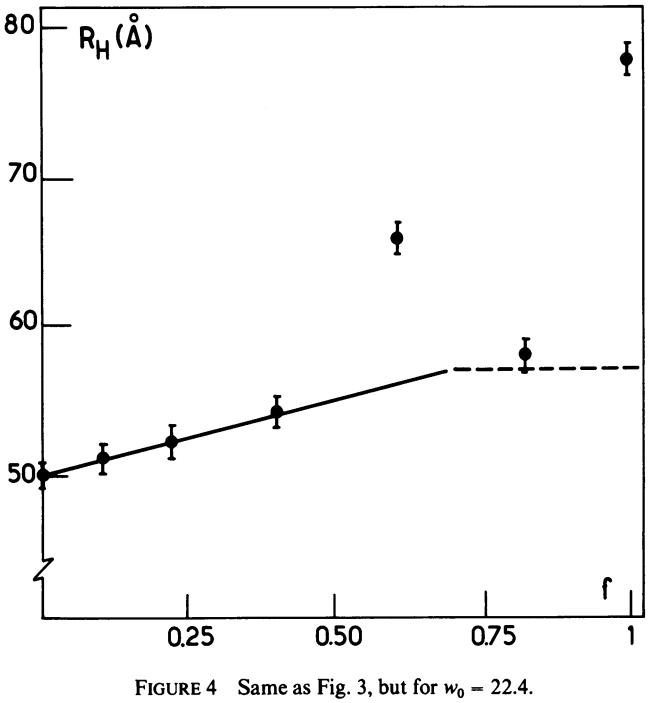

FIGURE 4 Same as Fig. 3, but for $w_0 = 22.4$.

Protein-containing Microemulsions at $w_0 = 22.4$

To extend the validity of the preceding analysis, we performed a series of experiments for $w_0 = 22.4$ in which the radius of the protein molecule is smaller than the radius of the water core of empty droplets. From the values $R_p = 24$ Å and $R_c = 50$ Å ($R_w = 38$ Å), it follows, without further assumption, from Eq. 1 a and b, that $R_f = 57$ Å. The value of n is then 1.4 and the limiting value of f is 0.7.

In Fig. 4, the calculated value of the mean radius is plotted vs. f and compared with experimental points. The agreement between both values is obviously excellent for $f < 0.6$. At higher f values, droplets including two protein molecules start to form as expected. Large aggregates were not observed at $w_0 = 22.4$, at least for $f \leq 1$. Indeed, droplets containing two protein molecules allow inclusion of all the protein present up to $f = 1.2$. Moreover, the probability of protein aggregation obviously decreases with decreasing n.

DISCUSSION

The water-shell model proposed by Bonner et al. (8), and some of its implications noted here, have been experimentally tested and found to be in excellent qualitative and quantitative agreement with the results obtained in this work by dynamic light scattering. The protein solubilization in the water core of droplets is satisfactorily described in the different situations, one in which the protein volume is larger ($w_0 = 5.6$) than the volume of the water core, the other in which it is smaller ($w_0 = 22.4$). In the former case, more than three empty droplets are necessary to build up a droplet of a sufficient size to accomodate one protein molecule.

The results reported here can be explained in part by the preferential binding of interfacial water by MBP, which is also suggested by the high solubility of the protein in microemulsion droplets at low water to surfactant ratios (20). This result is unexpected: indeed, optimal solubilization of other water-soluble proteins, so far reported, requires much higher w_0 values. Such an uncommon solubility seems to reflect an unusual interaction of MBP with the water core of microemulsion droplets that exhibit peculiar physical-chemical properties at such low w_0 values (2). Furthermore, the change of secondary structure observed upon insertion of MBP in microemulsion droplets (20) may lead to a more energetically favorable conformation, conferring a preferential solvation to the protein. These effects may mirror some of the properties in the aqueous myelin spaces where the protein interacts with both interfacial water and charged membrane surfaces.

Finally, it is interesting that the other major protein of CNS myelin, the Folch-Pi proteolipid, which is insoluble in aqueous solution, can also be solubilized in microemulsion droplets and also shows maximal solubilization at $w_0 = 5.6$ (6). There may thus be a similarity in the mechanism of interaction of these proteins with the interface provided by this system, probably bearing a resemblance to the native myelin environment.

APPENDIX

Calculation of the apparent radius, R, deduced from light-scattering measurements in a dispersion of droplets, some with a radius, R_c, the other ones with a radius, R_f.

For simplicity, the two types of droplets are assumed to be monodisperse.

The real correlation function is

$$\{\alpha\, e^{-at/R_e} + \beta\, e^{-at/R_f}\}^2,$$

with $a = kT\,q^2/6\pi\eta$. The relative weight of the two exponentials depends on the scattering power of droplets of radius, R_e and R_f, respectively. The most important factor in this scattering power is the influence of the size that scales as its sixth power. So

$$\alpha = (1 - nf)\, R_e^6$$
$$\beta = f\, R_f^6.$$

This correlation function is approximated by

$$\{(\alpha + \beta)e^{-at/R + a^2p/2(t/R)^2}\}^2.$$

Let $z = R_f/R_e$.

Taking the logarithm of the real correlation function and calculating its linear development, we can make an identification with the approximate formula. It leads to

$$R = R_e \frac{1 - nf + fz^6}{1 - nf + fz^5}$$

$$p = \left| \frac{z^2(z-1)}{1 - nf + z^5 f} \right|^2 f(1 - nf)$$

This quantity vanishes for $z = 0$ ($R_f = 0$), $z = 1$ ($R_e = R_f$), $z \to \infty$ ($R_e = 0$), $f = 0$ and $f = 1/n$, as expected. Its maximum value is 0.04 for $w_0 = 5.6$ and 0.004 for $w_0 = 22.4$, actually very low (much lower than the polydispersity of microemulsion droplets). Therefore, the validity of the procedure is justified.

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